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REVIEW

Rhizoremediation of metals: harnessing microbial communities

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Abstract With the increasing successful stories of decontamination, different strategies for metal remediation are gaining importance and popularization in developing countries. Rhizoremediation, is one such promising option that harnesses the impressive capabilities of microorganisms associated with roots to degrade organic pollutants and transform toxic metals. Since it is a plant based *in-situ* phytorestoration technique it is proven to be economical, efficient and easy to implement under field conditions.

Plants grown in metal contaminated sites harbor unique metal tolerant and resistant microbial communities in their rhizosphere. These rhizo-microflora secrete plant growth promoting substances, siderophores, phytochelators to alleviate metal toxicity, enhance the bioavailability of metals (phytoremediation) and complexation of metals (phytostabilisation). Selection of right bacteria/consortia and inoculation to seed/ roots of suitable plant species will widen the perspectives of rhizoremediation.

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Introduction

Worldwide contamination of soils with metals has posed a great threat to the human health as most of them are proven to be carcinogenic even at slightly higher concentrations. Metal contamination in India [1-3] is mainly due to industrial activities and it is estimated that about $3 billion are needed to remediate the metal contaminated sites alone in USA [4].

For the past few decades, three important strategies are used to treat contaminated soils i.e., *in-situ* immobilization of toxicants, *ex-situ* soil excavation and treatment and degradation/ detoxification of organic/ inorganic pollutants by physical, chemical or biological means. With the wide range of catabolic reactions mediated by microbes and its enzymes, bioremediation techniques till date are the most economical and ecofriendly strategies for organic and inorganic decontamination.

Rhizoremediation is an elegant form of bioremediation that seeks to harness light energy *via* plants to biostimulate pollutant degradation by the indigenous soil microbial community [5]. Microorganisms living in the rhizosphere on plant-derived substrates are better able to degrade or transform xenobiotics than are those in the bulk soil. This increased ability may be associated with the greater number of microorganisms or with the availability of growth-supporting substrates for co-metabolism.

The plant root system aerates the soil, distributes the rhizobacteria through soil and penetrates impermeable soil layers, solubilising the pollutants in soil-water and making it bioavailable to the plant and microbes. Researchers have exploited this symbiotic relationship for rhizoremediation of hazardous and xenobiotic compounds like PCBs [6], PAHs [7], TCE [8] and metal uptake [9,10]. As reviewed [11,12], rhizoremediation can be successfully used for restoration of contaminated sites by choosing right type of plant cultivar with right rhizobacteria or by inoculating efficient rhizobacterial strains on plant seeds/ roots. Also, the efficient rhizobacteria enabling hyperaccumulation could be potentially developed into an inoculum for commercial phytomining [13,14]. Various grasses and leguminous crops were used for rhizoremediation because of the profound root biomass [7]. The establishment of crop cover also improves the soil physical and chemical properties of contaminated soil and increase the contact microbes and contaminants [15].

Extensive literature exists on the role of rhizospheric bacteria on degradation of organic pollutants and also on beneficial rhizobacterial strains involved in biocontrol of soil borne pathogens [16] and biofertilisation [17]. However, science behind rhizosphere-microbes assisted metal transformations in soils needs to be explored further for implementation of this technique on a massive scale. This review highlights (i) the rhizosphere-microbial diversity of hyperaccumulators ii) microbes used in metal rhizoremediation and iii) recent advances in implementation of rhizoremediation techniques for metal removal from soils.

Rhizosphere microbial diversity of plants growing in metal rich soils

A variety of microbial forms can be found growing in rhizosphere micro-habitats. The microbial population in the rhizosphere depends on the composition of the root exudates, type of plant species, root type, age of the plant and also soil type. It is well documented that the rhizosphere is dominated with gram-negative bacteria, *Pseudomonads*. Rhizosphere bacteria associated with plants growing in metal contaminated soils harbor microbial populations that are i) resistant to metal ii) sequester/ bioaccumulate/ biosorb metals into cells and/ or iii) able to transform toxic metal species to non-toxic species by enzymes and exopolysaccharides produced.

It is evident that because of increased nutrients in rhizosphere the population of microorganisms in rhizosphere is several folds greater than the bulk soils as well as the overall microbial activity in rhizosphere. However, elevated concentrations of metals can inhibit overall population [18], microbial activities [19,20], microbial biomass [21], specific populations of bacteria or fungi [22] and cause a permanent shift in microbial community structure [23]. The inhibition is mostly significant in freshly contaminated soils. With time, a sequential change occurs in the diversity and composition of microbial communities and only the tolerant and resistant microbes thrive both in bulk as well as rhizosphere soils.

Table 1 Rhizospheric microbial communities associated with plants grown in metal rich soils

Plants	Microbe/ Microbial communities and their characteristics	Soil nature	Reference
Thlaspi goesingense	*Holophaga/ Acidobacterium division* and α-*proteobacteria, Methylobacterium mesophilicum, Sphingomonas*	Ni rich serpentine soils	24
T. caerulescens	Ni resistant bacteria predominant in rhizosphere than bulk soils		25
Alyssum murale	Ni resistant, siderophore and acid producing bacteria more in rhizosphere than bulk soils		26
	Sphingomonas macrogoltabidus, Microbacterium liquefaciens,M. arabinogalactanolyticum		27
A. bertolonii	Gram positive α-proteobacteria		28
Rinorea bengalensis Dichapelatum gelonioides ssp andamanicum	*Pseudomonas, Bacillus, Cupriavidus sp.*		10
Agrostis tenuis	*Arthrobacter, Ochrobactrum, Bacillus, Serratia sp* and AM fungi - *Acaulospora, Gigaspora*	As - contaminated cattle dip sites	29
Pteris vittata	Pseudomonads	As - contaminated soils	30
Phragmites sp.	Cu tolerant, exopolymer producing bacterial communities, predominantly, *Bacillus*	Cu - contaminated soils (Near Cu mines)	31

Heavy metal resistant rhizosphere bacteria are reported in soils rich in heavy metals and an unique microflora prevail in rhizosphere of hyperaccumulators grown in serpentine as well as contaminated soils (Table 1). The bacterial communities are predominant compared to fungi in these plants. The community structure of rhizosphere bacteria of *T.goesingense* identified by 16S rRNA sequence analysis showed that a higher percentage of bacteria belonging to *Holophaga/ Acidobacterium* division and α-*proteobacteria* were found. Rhizosphere associated endophytes belong mainly to genera *Methylobacterium*, resembling to *M.mesophilicum. Sphingomonas* is also predominant. All the isolates were resistant to Ni concentrations (5 - 12mM). All the bacteria were able to produce siderophores [24]. Similarly, phenotypic characterization of microbial populations of rhizosphere of *A.murale* reveal that Ni resistant, siderophore and acid producing bacteria were predominant in the rhizosphere soil compared to bulk soil [26]. The genetic diversity of the rhizosphere in *A.bertolonii* studied using T-RFLP analyses reveal that they belong mainly to gram positive and α- *proteobacteria* representatives [28]. The Ni hyperaccumulator growing in serpentine soils of India colonises Ni tolerant bacterial isolates belonging to *Pseudomonas, Bacillus* and *Cupriavidus.* Among this, *Cupriavidus pauculus* KPS 201(MTCC 6280) showed highest degree of Ni tolerance and maximum Ni uptake by the plants [10].

In metal contaminated soils, the rhizosphere microbes aid the uptake. In *Agrostis tenuis,* increased uptake was facilitated by rhizosphere microbes grown in As contaminated soil near cattle dip sites. The kikuyu grass and rainbow fern growing in this site had mixed infections of roots by *Acaulospora* and *Gigaspora*. The bacteria belong to the genus *Arthrobacter* and *Ochrobactrum* [29]. The *Phragmites* sp. grown in Cu contaminated soils has Cu-resistant bacterial communities in rhizosphere. Compared to bulk soils, the bacteria in rhizosphere was prompted by Cu stimuli and produce exopolymers in large quantities that helps in detoxification of Cu to both bacteria and plants [31].

The changes in concentration of the same metal (low/ high) also changed the microbial diversity and tolerance level in rhizobacteria of *Brassica juncea* [32] and *Diplachne fusca* [18]. Multiple metal resistance in rhizobacteria seems to be the rule. Bacteria colonizing *Alyssum* sp. tolerate Ni, Co, Cu. Strains showing multiple metal tolerance are more predominant than mono-tolerance [18]. About 107 bacterial strains were checked for multimetal tolerance and all of them can tolerate more than six metals. In general rhizospheric bacteria are metal tolerant and/or resistant to a variety of metals. Most of them possess certain mechanisms to cope up with very high concentration of metals. These special traits of rhizospheric microbes are used in rhizoremediation techniques in conjunction with right plant species.

Rhizobacteria in metal bioremediation

For effective bioremediation of metal contaminated soils, several organisms have been utilized isolated from varied environments [33,34]. Microbial isolates from rhizosphere can also be effectively harnessed for bioremediation of contaminated environments. All the PGPR strains can be used for bioremediation of metals.

Plant growth-promoting rhizobacteria include a diverse group of free-living soil bacteria that can improve host plant growth and development in heavy metal contaminated soils by mitigating toxic effects of heavy metals on the plants [35]. A list of PGPR associated with plants grown in metal contaminated soils like *Azotobacter chroococcum* HKN-5, *Bacillus megaterium* HKP-1, *Bacillus mucilaginosus* HKK-1, *Bacillus subtilis* SJ-101, *Brevundimonas* sp. KR013, *Pseudomonas fluorescens* CR3, *Rhizobium leguminosarum bv. trifolii NZP561, Kluyvera ascorbata* SUD165 [33] are used in bioremediation. The rhizobacteria associated with hyperaccumulators, *Bacillus subtilis, Bacillus pumilus, Pseudomonas pseudoalcaligenes* and *Brevibacterium halotolerans* are also widely used in bio- and rhizo-remediation of multimetal contaminated sites [36].

Nickel tolerant rhizosphere bacterial isolates belonging to genus *Pseudomonas*, *Bacillus* and *Cupriavidus* from *Rinorea bengalensis* and *Dichapetalum gelonioides* ssp. *Andamanicum* were capable of accumulating nickel (209.5–224.0 μM Ni g [−1] protein) from aqueous solution. These isolates are also capable of tolerating high concentration of Ni and possess nickel uptake potential too. Hence, can be effectively harnessed for bioremediation of Ni contaminates sites [10]. Compared to a single strain, group of bacterial cultures can be very effective. Chen and Cutright [9] utilized a rhizobial microbial consortium for treating an aqueous solution containing 600mg/L of Cd, Cr and Ni. The consortium was resistant to metal toxicity and facilitated reduction in aqueous metal concentration with selectivity of Cr > Cd >Ni.

Rhizoremediation of metals

The diverse microbial communities in rhizosphere, the interactions of these microbes among themselves and with plants determine the extent of rhizoremediation. The rhizobacteria is used or manipulated with three main objectives for remediation of metal contaminated soils a) hyperaccumulation of metals in plants b) reducing the uptake of

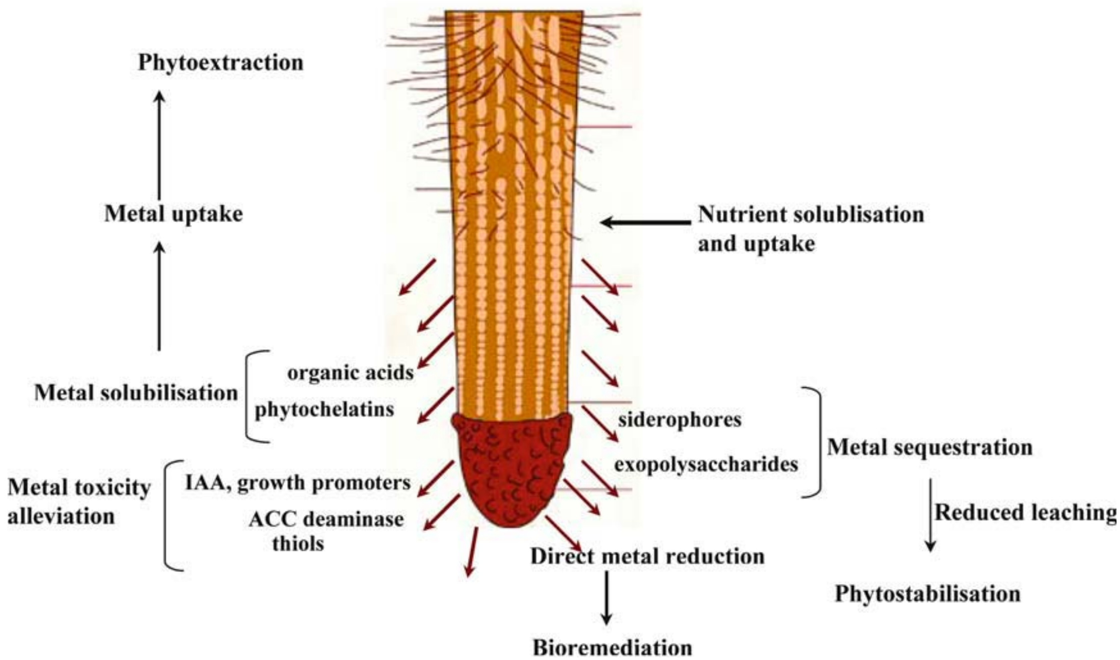

Fig. 1

metals and c) *in-situ* stabilization of the metals as organo-complexes.

Increased metal uptake in hyperaccumulators is aided by changes in the rhizosphere and rhizobacterial secretions. The chemical condition of the rhizosphere differs from bulk soil as a consequence of various processes induced by plants roots as well as by rhizobacteria [37] (Fig. 1) like secretion of organic acids followed by reduction in pH, production of siderophores, phytochelains, amino acids and ACC deaminase.

As seen in the Table 2 [38–51], predominantly rhizobacteria increased the dissolution of metals like Zn, Ni and Cu thereby increasing the dissolution of metal and more uptake by plants. Delorme et al [52] reported that soil acidification increased the metal ion mobility in *T.caerulescens*. Similarly, the accumulation of Hg. It was observed that the pH in the rhizosphere soil of the Cu accumulating plant species (*Elsholtzia*) was significantly lower than in the bulk soil when plants were grown in Cu and other metal contaminated soil under field experiment conditions [53].

Pseudomonas maltophilia was shown to reduce the mobile and toxic Cr [6+] to nontoxic and immobile Cr [3+], and also to minimize environmental mobility of other toxic ions (Hg, Pb, Cd) [54]. Chromium-resistant pseudomonads, isolated from paint industry effluents, were able to stimulate seed germination and growth of *Triticum aestivum* in the presence of potassium bichromate [55]. In this case, the bacterial enhancement of seedling growth was associated with reduced chromium uptake.

Abou-Shanab et al [13] studied the effect of rhizobacteria that facilitated the release of Ni and more accumulation in *A.murale*. In *B. napus*, the inoculation of Cd resistant rhizobacteria increases the accumulation of Cd [47].

Rhizobacteria produce siderophores that have an important role in sequestering metals [56]and has more affinity to plants. Microbial siderophores are used as metal chelating agents that regulate the availability of iron in plant rhizosphere. This helps the plants to alleviate the toxicity of metals. Since As and P are chemical analogues, increased As plant uptake was recorded in *P.vittata* [30]. Under metal stress conditions, phytoharmones (IAA and ethylene) are released and results in increased uptake of metal ions [57]. Some PGPR contain the enzyme ACC deaminase that helps in reducing the impact of ethylene on root growth [58].

Thlaspi caerulescens has a remarkable ability to hyperaccumulate Zn from soils containing mostly nonlabile Zn. The addition of bacteria to surface-sterilized seeds of *T. caerulescens* sown in autoclaved soil increased the Zn concentration in shoots 2-fold as compared to axenic controls; the total accumulation of Zn was enhanced 4-fold [42,64]. Heavy metals stimulate the production of siderophores eg., under cadmium stress the synthesis of phytoharmones are triggered that ended up in higher uptake of metals [59]. Abou-Shanab et al [18]investigated the correlation between

Table 2 Rhizosphere-microbes aided metal remediation

Metal	Associated plant	Microbial action in rhizosphere aiding metal uptake	Changes in plants	Reference
Zn	*Thlaspi caerulescens*	Bacterially mediated dissolution of Zn from non labile phase	4 fold more uptake compared to anexic control	38
Ni	*Alyssum murale*	Bacterial Ni solubilisation	Increased Ni uptake into the shoot (17-32%)	27
	Brassica campestris *Lycopersicon esculentum*	*Kluyvera ascorbata* SUD165 Siderophore production and ACC deaminase activity	Reduced uptake and reduces toxicity	39
Se	*Brassica juncea L*	Bacteria volatilizes Se into nontoxic forms, such as dimethylselenide	35% of plant Se volatilization and 70% of plant tissue accumulation	40
As	*Pteris vittata*	Mycorrhizae increased the amount of P transporters at hyphae level for As uptake Phenolic defense system (formation of thiol like glutatuhione)	More accumulation and increased shoot biomass	41,42 43
		Mycorrhization *Glomus mosseae* or *Gigaspora margarita*	Increased pinnae dry weight, leaf area and reduced root concentration	44
Cu	*Elsholtzia splendens*	Dissolution of Cu by addition of rhizobacterial strain MS12 and ampicillin 0.1mg/g	2.2-fold and 2.5-fold increase in Cu accumulation in the shoots and roots	45
Cd	*Trifolium repens*	Coinoculation of *Brevibacillus* sp. and AM fungus	Increased Cd uptake (37%)	46
	Brassica napus	Cadmium resistant bacterial strains inoculated to plants. (Indole acetic acid as auxin produced by the isolates for tolerance)	Increased Cd content (16-74%	47
	Arabidopsis sinicus	Inoculation of recombinant *Mesorhizobium huakuii* subsp. rengei B3	Increased Cd accumulation in nodules (1.5 fold)	48
Multi-metals	*Zea mays*	Inoculation of *Brevibacterium haloterans*	Pb (0.2 g kg^{-1}), Zn (4 g kg^{-1}) and Cu (2 g kg^{-1}) were accumulated in shoots	36
		Mycorrhizae bound metals to organic matter and increases uptake	Cu (+5%), Zn (+23%) and Pb (+3%)	49
	Helianthus annus	Inoculation of Engineered Rhizobacteria (*Pseudomonas putida* 06909 with metal binding peptide EC20)	Decrease in Cd phytotoxicity; 40% increase in Cd accumulation in the plant root	50
TCE and heavy metals		EC20, was introduced into rhizobacteria *Pseudomonas* strain Pb2-1 and *Rhizobium* strain10320D	Sixfold higher Cd accumulation than non-engineered strains in the presence of 16 mM $CdCl_2$.	51

metal resistance and metal mobilization abilities of rhizobacteria under heavy metals stress. The highest incidence of the biochemical activity of isolates and metal resistance was recorded for: phosphate solubilizers with Cr, Zn and Pb (92.5%, 82.2% and 68.2%), respectively followed by siderophore producers and acid formers. This implies that phosphate solubilization is not only the mechanism adopted by bacteria towards metals in soil.

The effect of adding *K. ascorbata* SUD165, a plant growth-promoting bacterium, to canola or tomato seeds before the seeds germinate, was examined in the presence of inhibitory concentrations of Ni^{2+}. Addition of this bacterial strain significantly decreased the toxicity of the added nickel [39]. Addition of a bacterial strain *P. maltophila* was shown to reduce the mobile and toxic Cr(VI) to immobile and nontoxic Cr(III) thereby minimizing the mobility of metal ions [20]. Results also reveal that in long-term chrome contaminated sites, the complete microbial reduction of Cr(VI) is challenging [60].

The association of mycorrhiza with brake fern, *Pteris vittata* (hyperaccumulator for As) is well studied. Al-Agely et al [41]studied the effect of increasing levels of As and P on the fern infected with mycorrhiza. The mycorrhiza tolerated elevated concentrations of As as well as increased dry biomass of the fern. Leung et al [61] also reported that the addition of rhizofungi enhanced the uptake and accumulation of As in *P. vittata*. Under the condition of 100 mg As per kg soil, non-colonized plants accumulated 60.4 mg As kg^{-1} while plants colonized by arbuscular mycorrhizal fungi (AMF) isolated from an As mine accumulated 88.1 g As kg^{-1} and also enhanced plant growth. On the other hand, Trotta et al [44] found that in the same plant species, *P. vittata*, rhizofungi increased plant growth only in the above ground parts but reduced root As concentration without any effect on frond concentration, therefore resulting in a larger As translocation factor. Moreover, in U and As-contaminated soil, Chen et al [62] found that rhizofungi depressed growth of *P. vittata* particularly at the early stages and had no effect on As concentration in this plant. These results indicated that the effects of rhizofungi on As uptake is inconsistent even though the same plant species was used.

The presence of mycorrhiza likely increased the amount of P transporters at hyphae level. A phenolic defense mechanism is also reported wherein the formation of thiol like glutothione is induced as the concentration of As increased. This alleviates the toxicity of plants [43]. More research is needed to investigate further the low molecular weight thiols [42]. Research is also underway to study the genetic and functional characters of the rhizosphere microbial communities in *P.vittata*. An As resistant Pseudomonad has been isolated and its role in solubilisation of phytic acid is studied further [30].

Chelate-assisted phytoremediation has been proposed as an effective tool for the extraction of heavy metals from soil by plants. Chen et al [63] reported that chelate addition facilitated phytoremediation of soil Cu without inhibiting the microbial communities. Cocultivation of crops also enhances metal uptake. Rhizospheric diversity was considerably reduced in the rhizospheres of monocultures of *L.perenne* and *T. repens* compared to the diversity in bulk soil [64]. The greater diversity of plant species may be responsible in part for the greater bacterial diversity in the bulk soils. The hyperaccumulator plants are also grown along with non-hyperaccumulators to enhance the heavy metal uptake by intermingling the roots and hence, the colonization of efficient rhizobacteria.

Engineered rhizobacteria for metal uptake

Biosorption using microbially produced synthetic phytochelatins as been shown to be a promising technique for ameliorating heavy-metal contamination. Bacteria such as *Escherichia coli* and *Moraxella* sp. expressing EC20 (with 20 cysteines) on the cell surface or intracellularly have been shown to accumulate up to 25-fold more cadmium [65]or mercury [66]than the wild-type strain.

Recombinant *Mesorhizobium huakii* by incorporating the gene (phytochelatinsynthase) from *Arabidopsis thaliana* into *M.* sub sp *rengei* B3 increased 1.5 fold more Cd accumulation in *Astralagus sinicus* [48].

Combining the advantages of microbe plant symbiosis within the plant rhizosphere is an effective cleanup technology. Inoculation of sunflower roots with the engineered rhizobacterium (metal-binding peptide (EC20) in a rhizobacterium, *Pseudomonas putida* 06909) resulted in marked decrease in Cd phytotoxicity and increase in Cd accumulation [49].

However, one major obstacle for utilizing these engineered microbes is sustaining the recombinant bacteria population in soil, with various environmental conditions and competition from native bacterial populations.

Challenges

When evaluating the effect of rhizobacteria on remediation of contaminated soils, it is very certain that many bacteria can eventually find a use in bioremediation. Treating the seeds with rhizobacteria has opened up new avenues in the area of rhizoremediation and can contribute to the restoration of polluted sites. However, not many reports exist on the utilization of this technique on a massive scale. Few more challenges facing the field of rhizoremediation is the availability of suitable methods to study *in-situ* metal transformation processes and also molecular approaches. Standardised methods were available for organics, not many for inorganics. Further, the studies on the selection of more rhizobacteria, bacteria-plant combination and effective means to sustain and proliferate the rhizobacteria in the roots will be helpful to formulate suite of remediation strategies.

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